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# Shear Stress Theory and Small-for-Size Graft in Adult Living Related Liver Transplantation

Y. Sato, T. Ichida, S. Yamamoto, K. Hirano, T. Kobayashi, H. Oya, H. Nakatsuka, T. Watanabe, and K. Hatakeyama

SIZE mismatch is the greatest obstacle in adult living related donor liver transplantation (ALRDLT). We have reported that acute elevations of portal hypertension, reflecting wall shear stress against the portal vein and sinusoids, trigger liver regeneration following partial hepatectomy and that excessive shear stress induces liver injury after massive hepatectomy.[1,2] We have also reported that splenic arterial ligation and splenectomy prevent the liver injury by reducing excessive portal hypertension after major hepatectomy.[3] In this study, we investigated the relation between posttransplant portal hypertension and small-for-size grafts in ALRDLT.

## MATERIALS AND METHODS

We examined 15 patients transplanted from March 1999 to April 2001. The patients were divided into three groups: Group I, graft volume/recipient body weight (GV/RBW) ratio ≥1.0; Group II, GV/RBW ratio <1.0; and Group III, recent cases with measured portal pressure after ALRDLT and control of portal pressure below 25 cm $H_2O$. The patients in Group III were administered intraportal prostaglandin E1 and insulin after operation with examination of the liver function and immediate portal pressure following ALRDLT.

## RESULTS

Peak total bilirubin levels of the three groups after ALRDLT were 7.6 ± 3.3 mg/dL, 17.4 ± 12.9 mg/dL, and 9.3 ± 4.0 mg/dL, respectively. Peak GPT levels of three groups were 466 ± 402 IU/L, 166 ± 105 IU/L, and 179 ± 99 IU/L, respectively. There were no statistical significances in *AKBR* between Group I and Group II. During postoperative hyperbilirubinemia, direct bilirubin was the dominant fraction, especially in Group II. Interestingly, a patient who underwent auxiliary partial orthotopic liver transplantation due to metabolic liver disease did not display portal hypertension (11 cm $H_2O$) and the direct bilirubin did not increase. The portal pressure of three patients after ALRDLT increased above 30 cm $H_2O$ despite a right lobe graft. Splenectomy was performed in these patients The portal pressure after splenectomy decreased below 25 cm $H_2O$. The postoperative courses of the splenectomized patients were uneventful. On the other hand, the portal pressures of the patient whose GV/RBW ratio was 0.55 was 19.5 cm $H_2O$ after ALRDLT.

## DISCUSSION

Excessive portal pressure after hepatectomy or ALRDLT would result in liver failure. Our animal data,[4] in which portosystemic shunts were created by subcutaneous splenic transposition in hosts after 95% massive hepatectomy showed inhibited liver injury and prolonged survival, findings that support our hypothesis. There was a discrepancy between posttransplant portal pressure and GV/RBW ratio. Measurement of portal pressures after ALRDLT may be useful to recognize this discrepancy. Splenectomy or splenic arterial ligation reduces excessive portal hypertension after ALRDLT and may prevent liver injury due to excessive portal hypertension, including damage to the transport of direct bilirubin.

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From the Division of Digestive and General Surgery (Y.S., S.Y., K.H., T.K., H.O., H.N., T.W., K.H.), Gastroenterology and Hepatology (T.I.), Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan.

Address reprint requests to Dr Yoshinobu Sato, Division of Digestive and General Surgery, Gastroenterology and Hepatology, Niigata University Graduate School of Medical and Dental Sciences, 1-757 Asahimachi-dori, Niigata, 951-8510 Japan.

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# Analysis of Microchimerism in Peripheral Blood by Short Tandem Repeat Sequences Immediately After Living Related Liver Transplantation

Y. Sato, T. Ichida, S. Yamamoto, H. Oya, H. Nakatsuka, T. Kobayashi, T. Watanabe, H. Kameyama, and K. Hatakeyama

MICROCHIMERISM[1,2] in organ transplantation has been proposed to determine transplant outcome; however, there are no investigations concerning early chimerism after liver transplantation. We have reported that intrahepatic leukocytes are washed out of the graft liver due to the actions of excessive shear stress caused by the preservation solution and the increased portal flow.[3-5] Therefore, in the present study, we investigated the microchimerism during the early period following adult living related donor liver transplantation (ALRDLT).

## MATERIALS AND METHODS

Three consecutive patients who underwent ALRDLT beginning in July 2000 were studied to assess peripheral blood microchimerism using a polymerase chain reaction (PCR)-based method of microsatellite analysis of highly polymorphic short tandem repeat sequences (STRs) that detect donor-type cells, which were counted by GeneScan3.1 (ABI PRISM).

## RESULTS

Donor-type cells were not detected 2 days after ALRDLT in any patients. However, immediately after liver transplantation (Fig 1), over 10% of all peripheral blood leukocytes were of the donor type (mean: 13.4% ± 2.4%).

From the Division of Digestive and General Surgery (Y.S., S.Y., H.O., H.N., T.K., T.W., H.K.) and Gastroenterology and Hepatology (T.I.), Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan.

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Address reprint requests to Dr Yoshinobu Sato, Division of Digestive and General Surgery, Gastroenterology and Hepatology, Niigata University Graduate School of Medical and Dental Sciences, 1-757 Asahimachi-dori, Niigata, 951-8510 Japan.

Result of donor and recipient STR locus

| STR locus | Recipient Pre op | Donor |
|---|---|---|
| CSF1PO | 12, 8 | 12, 8 |
| TPOX | 8 | 8 |
| TH01 | 9, 8 | 8, 7 |

Results of chimerism in peripheral blood

| | Immediatly after implantation | 12 hours after implantation |
|---|---|---|
| CSF1PO | 12, 8 | 12, 8 |
| TPOX | 8 | 8 |
| TH01 | 9, 8, 7 | 9, 8 |

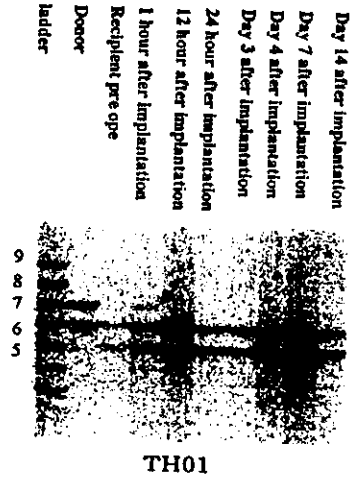


**Fig 1.** Analysis of microchimerism in peripheral blood following ALRDLT by short tandem repeat method. Leukocytes could be divided into donor type or recipient type by STRs locus 7 (underline) using TH01. The percentage of donor-type leukocytes was 13.6% 1 hour after the implantation.

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## DISCUSSION

Since microchimerism was reported by Starzl et al,[1] the impact of this phenomenon on donor-specific graft tolerance has been controversial. Norris et al.[2] reported that donor microchimerism following liver transplantation is an infrequent event and that generation of graft acceptance is independent of microchimerism.

We reported that intrahepatic leukocytes changed from donor type to recipient type within 1 week in over 95% of hosts.[6] Therefore, in this study, we examined early alloimmune reactions following ALRDLT. Our data demonstrate that donor-type cells are eliminated from the periperal blood within 1 or 2 days after ALRDLT, suggesting that an alloimmune reaction occurs immediately after transplantation. Furthermore, a fair number of donor-type cells present donor antigens from the grafted liver to the systemic circulation immediately after ALRDLT. This antigen-presenting system may participate in graft acceptance.

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# Reevaluation of Prognostic Factors for Survival after Liver Resection in Patients with Hepatocellular Carcinoma in a Japanese Nationwide Survey

Iwao Ikai, M.D.[1]
Shigeki Arii, M.D.[2]
Masamichi Kojiro, M.D.[3]
Takafumi Ichida, M.D.[4]
Masatoshi Makuuchi, M.D.[5]
Yutaka Matsuyama, Ph.D.[6]
Yasuni Nakanuma, M.D.[7]
Kiwamu Okita, M.D.[8]
Masao Omata, M.D.[9]
Kenichi Takayasu, M.D.[10]
Yoshio Yamaoka, M.D.[1]
for The Liver Cancer Study Group of Japan

[1] Department of Gastroenterological Surgery, Kyoto University Graduate School of Medicine, Kyoto, Japan.

[2] Department of Hepato-Biliary-Pancreatic Surgery, Tokyo Medical and Dental University, Graduate School of Medicine, Tokyo, Japan.

[3] Department of Pathology, Kurume University School of Medicine, Kurume, Japan.

[4] Department of Gastroenterology, Juntendo University School of Medicine, Tokyo, Japan.

[5] Hepato-Biliary-Pancreatic Surgery Division, Department of Surgery, Graduate School of Medicine, University of Tokyo, Tokyo, Japan.

[6] Department of Biostatistics, School of Health Sciences and Nursing, University of Tokyo, Tokyo, Japan.

[7] Department of Human Pathology, Kanazawa University Graduate School of Medicine, Kanazawa, Japan.

[8] Department of Gastroenterology and Hepatology, Yamaguchi University School of Medicine, Ube, Japan.

[9] Department of Gastroenterology, Graduate School of Medicine, University of Tokyo, Tokyo, Japan.

[10] Department of Diagnostic Radiology, National Cancer Center Hospital, Tokyo, Japan.

Address for reprints: Iwao Ikai, M.D., The Liver Cancer Study Group of Japan, 403 Bear House, 40, Sanno-cho, Shogoin, Sakyo-ku, Kyoto, 606-8392, Japan; Fax: (011) 81 757525411; E-mail: ikai@kuhp.kyoto-u.ac.jp

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**BACKGROUND.** Advances in the diagnosis and surgical treatment of hepatocellular carcinoma (HCC) have improved the prognosis for patients with HCC who undergo liver resection. The objective of this study was to evaluate prognostic predictors for patients with HCC who underwent liver resection in a Japanese nationwide data base.

**METHODS.** In this study, the authors analyzed 12,118 patients with HCC in a Japanese nationwide data base who underwent liver resection between 1990 and 1999 and compared them with a previous analysis of patients between 1982 and 1989. All patients were evaluated for prognostic factors.

**RESULTS.** During the last decade, the increases in patients who were without hepatitis B virus surface antigen, who had small tumors, and who had portal vein invasion were noted. The 5-year overall survival rates for patients with HCC improved to 50.5%, compared with < 40% in the previous analysis. A multivariate analysis using a stratified Cox proportional hazards model according to associated liver disease indicated that age, degree of liver damage, $\alpha$-fetoprotein level, maximal tumor dimension, number of tumors, intrahepatic extent of tumor, extrahepatic metastasis, portal vein invasion, hepatic vein invasion, surgical curability, and free surgical margins were independent prognostic predictors for patients with HCC. Operative mortality decreased from 2.3% in 1990–1991 to 0.6% in 1998–1999.

**CONCLUSIONS.** Outcomes and operative mortality rates in patients with HCC improved during the last decade. Age, degree of liver damage, $\alpha$-fetoprotein level, maximal tumor dimension, number of tumors, intrahepatic extent of tumor, extrahepatic metastasis, portal vein invasion, hepatic vein invasion, surgical curability, and free surgical margins were prognostic factors for patients with HCC who underwent liver resection. *Cancer* 2004;101:796–802.


**KEYWORDS: hepatocellular carcinoma, prognostic factor, liver resection, nationwide survey data base.**


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Hepatocellular carcinoma (HCC) is a common malignancy in Japan. Recent progress in the diagnosis and treatment of HCC has improved patient outcomes. With earlier diagnosis and tumors detected at an early stage, there is substantially increased survival, and curative surgical resection possible.[1–3] Advances in surgical techniques and perioperative management have led to a decline in the operative morbidity and mortality of patients who have HCC with chronic liver disease. However, long-term survival remains unsatisfactory.

The Liver Cancer Study Group of Japan (LCSGJ) has been conducting a nationwide survey of patients with primary liver carcinoma since 1965 to evaluate epidemiologic and clinical characteristics, histopathologic features, diagnosis, treatment modalities, and outcomes. In 1994, the LCSGJ reported on predictive factors for long-term prognosis after liver resection for patients with HCC between 1982 and 1989 in Japan.[2] Since that study was published, new imaging methods and laboratory tools have been introduced for the diagnosis of HCC.[4,5] In addition, it is recognized now that hepatitis C viral infection confers a high risk for HCC.[6] In the 1990s, a screening program for HCC was initiated, and several new therapeutic modalities for HCC, such as thermal ablation therapy and locoregional infusion chemotherapy through the hepatic artery, have been applied. Liver transplantation also has become an effective treatment for patients with HCC who have severe liver dysfunction.[7] However, new strategies for the treatment of patients with HCC still need to be established.

We reevaluated prognostic predictors for patients with HCC in a large-scale data base, taking into consideration recent advances in the diagnosis and treatment of HCC. In this study, we analyzed a Japanese nationwide data base between 1990 and 1999 to reevaluate prognostic factors for patients with HCC who underwent liver resection and compared the findings with data from our previous report.[2]

## MATERIALS AND METHODS

In the nationwide follow-up survey of primary hepatic carcinoma conducted by the LCSGJ, patients with primary malignant liver tumors who were diagnosed with imaging studies, preoperative clinical data, and/or histopathologic studies at approximately 800 institutions in Japan were registered every 2 years, and registered patients were followed prospectively. In this data base, there were 50,267 patients who were diagnosed with HCC between 1990 and 1999. Among them, we enrolled 12,118 patients with HCC who underwent liver resection between January, 1990 and December, 1999. Follow-up ended on December 31, 1999. The median follow-up was 21.5 months (range, 0.03–119.7 months), the mean patient age was 62.5 years, and the male:female ratio was 3.73:1.0.

TABLE 1
Degree of Liver Damage

| Item | Degree of liver damage[a] | | |
|---|---|---|---|
| | A | B | C |
| Ascites | None | Controllable | Uncontrollable |
| Serum bilirubin (mg/dL) | < 2.0 | 2.0–3.0 | > 3.0 |
| Serum albumin (g/dL) | > 3.5 | 3.0–3.5 | > 3.0 |
| $ICGR_{15}$ (%) | < 15 | 15–40 | > 40 |
| Prothrombin activity (%) | > 80 | 50–80 | < 50 |

$ICGR_{15}$ indocyanine green retention rate at 15 minutes.
[a] The severity of each finding is evaluated separately. Degree of liver damage is recorded as A, B, or C, based on the highest grade that contained at least two findings.

In the data base, 21 clinicopathologic and biologic variables were selected from LCSGJ questionnaires that were used to survey patients with HCC. Patients were then stratified by gender, age, history of blood transfusion, hepatitis B virus surface antigen (HBs-Ag) status, hepatitis C virus antibody (HCV-Ab) status, degree of liver damage, preoperative serum $\alpha$-fetoprotein (AFP) and protein induced by vitamin K absence or antagonist-II (PIVKA-II) levels, maximal tumor dimension, number of tumors, intrahepatic extent of tumor, extrahepatic metastasis (including lymph node metastasis indicated in the preoperative imaging studies or operative findings), growth appearance (expansive growth or infiltrative growth), capsular formation, septum formation, portal vein invasion, hepatic vein invasion, bile duct invasion, surgical curability of liver resection, free surgical margins, and histologic-associated liver disease (normal liver, chronic hepatitis, and cirrhosis). The degree of liver damage was classified as A, B, and C (also called clinical Stages I, II, and III, respectively in the *Classification of Primary Liver Cancer* by the LCSGJ[8]) and was defined by preoperative measurements of ascites, serum bilirubin level, serum albumin level, indocyanine green retention rate at 15 minutes, and prothrombin activity (Table 1). Intrahepatic extent of tumor also was defined in the *Classification of Primary Liver Cancer* by the LCSGJ.[8] We defined a free surgical margin as a distance of 1 cm between the cut surface and the tumor edge in the resected specimen. Surgical curability was defined by the LCSGJ as follow: Absolute curative resection included live resection with 1 cm of free surgical margin in patients with Stage I disease; relative curative resection included live resection without 1 cm of free surgical margin but with the excised tumor tissue in patients with Stage I disease or liver resection with 1

cm of free surgical margin in patients with Stage II or III disease (in either instance, no tumor thrombi may remain in the portal vein, hepatic vein, or bile duct in images of the remnant liver); relative noncurative resection, in which all macroscopic tumor tissue is removed; and absolute noncurative resection, which is liver resection with part of the macroscopic tumor tissue remaining. Capsular infiltration was excluded, because this factor was determined only in patients who had HCC with capsular formation; and, in 25% of our patients, capsular formation was absent.

Overall, cumulative survival rates were obtained using the Kaplan–Meier method. The differences in survival between the groups were compared using the log-rank test. The starting point for calculating survival was the date of surgery, and the endpoint was the date of death. All deaths, including operative deaths, were considered the endpoint. Patients who remained alive on December 31, 1999, were censored. After the univariate analysis of the factors affecting survival, only significant variables except PIVKA-II were used in the multivariate analysis using the stratified Cox proportional hazards model, because data records of PIVKA-II were not complete. In the Cox model, baseline hazards were stratified by underlying liver disease, because the underlying liver disease affected survival in the univariate analysis and crossed the Kaplan–Meier curves. It was expected that this stratified Cox model would be more powerful than the subgroup analysis by underlying liver disease. The data derived from 7056 patients with complete records were used in the multivariate analysis. $P$ values $< 0.05$ were considered statistically significant. Statistical analysis was carried out using SAS software (version 8.02; SAS Inc., Cary, NC).

## RESULTS

The 1-year, 3-year, and 5-year overall survival rates for patients with HCC were 85.3%, 67.0%, and 50.5%, respectively (Fig. 1). The 5-year survival rate was 10% greater than the rate in our previous report, in which the 5-year survival rate was < 40%.[2] Categorization of variables, patient ratios, and the 3-year and 5-year survival rates are shown in Table 2. Compared with our previous report, the proportion of patients age > 60 years increased from 46% to 66%. The percentage of patients with positive HBs-Ag status decreased from 25% to 20%; and the percentage of patients with positive HCV-Ab status, which was not examined in the previous study, was > 60%. The development of diagnostic imaging studies made it possible to detect small tumors, so that patients with tumor s that measured ≤ 2 cm in greatest dimension increased from 16% to 21%, and their 5-year survival rate improved from 53%

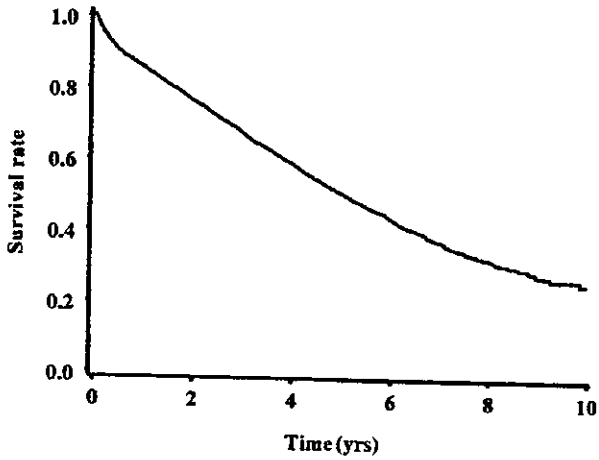


**FIGURE 1.** Survival curve for patients with hepatocellular carcinoma (HCC) who underwent liver resection between 1990 and 1999 ($n$ = 12,118 patients). The 1-year, 3-year, and 5-year overall survival rates for patients with HCC patients were 85.3%, 67.0%, and 50.5%, respectively.

to 66%. This early detection of small HCC tumors led to an increase in patients with AFP levels < 20 ng/mL from 32% to 43%. The percentage of patients who had solitary tumors was similar to the percentage in our previous report; however, their 5-year survival rate increased from 41% to 56%. Improved surgical techniques have made it possible to resect advanced hepatic tumors. The proportion of patients with portal vein invasion who underwent liver resection increased from 15% to 24%. The indications for surgical treatment of advanced HCC have been expanding.

In the univariate analysis, there were significant differences in survival among the groups stratified by age, degree of liver damage, serum level of AFP and PIVKA-II levels, maximal tumor dimension, number of tumors, intrahepatic extent of tumors, extrahepatic metastasis, growth appearance, capsular formation, septum formation, portal vein invasion, hepatic vein invasion, bile duct invasion, surgical curability, free surgical margin, and associated liver disease (Table 2). Compared with our previous report, the survival of patients with negative HBs-Ag status, including patients with HCV-Ab, was no better than the survival of patients with negative HBs-Ag status. There also were no significant differences in survival between patients with negative and positive HCV-Ab status.

The multivariate analysis using a Cox proportional hazards model stratified by associated liver disease indicated that age, degree of liver damage, AFP level, maximal tumor dimension, number of tumors, intrahepatic extent of tumors, extrahepatic metastasis, portal vein invasion, hepatic vein invasion, surgical curability, and free surgical margin were independent

TABLE 2
Univariate Analysis of Patients with Hepatocellular Carcinoma

| Variable | No. of patients (%) | Survival rate (%) 3 Yrs | 5 Yrs | P value |
|---|---|---|---|---|
| Age | | | | |
| < 60 yrs | 4097 (33.8) | 67.3 | 53.7 | 0.01 |
| ≥ 60 yrs | 8021 (66.2) | 66.8 | 48.4 | — |
| Gender | | | | |
| Male | 9550 (78.9) | 67.1 | 50.3 | 0.8 |
| Female | 2561 (21.1) | 66.5 | 50.9 | — |
| History of blood transfusion | | | | |
| Present | 2595 (25.9) | 67.1 | 49.4 | 0.25 |
| Absent | 7435 (74.1) | 67.7 | 51.5 | — |
| Hepatitis B surface antigen | | | | |
| Positive | 2295 (19.8) | 64.9 | 53.0 | 0.57 |
| Negative | 9276 (80.2) | 67.6 | 50.8 | — |
| Hepatitis C virus antibody | | | | |
| Positive | 7577 (67.3) | 68.4 | 49.8 | 0.25 |
| Negative | 3676 (32.7) | 66.6 | 53.6 | — |
| Degree of liver damage | | | | |
| A | 7362 (66.4) | 72.3 | 56.1 | 0.0001 |
| B | 3299 (29.7) | 60.3 | 42.9 | — |
| C | 429 (3.9) | 44.2 | 26.7 | — |
| α-Fetoprotein | | | | |
| < 20 ng/mL | 4980 (42.9) | 77.1 | 61.5 | 0.0001 |
| 21–200 ng/mL | 3296 (28.4) | 67.2 | 47.0 | — |
| 201–1000 ng/mL | 1504 (13.0) | 58.7 | 41.5 | — |
| 1001–1000 ng/mL | 1187 (10.2) | 52.1 | 37.7 | — |
| ≥ 10,001 ng/mL | 637 (5.5) | 40.3 | 33.1 | — |
| PIVKA-II | | | | |
| < 100 mAU/mL | 5352 (62.0) | 75.3 | 57.3 | 0.0001 |
| 100–299 mAU/mL | 839 (9.7) | 67.8 | 53.9 | — |
| 300–499 mAU/mL | 421 (4.9) | 59.1 | 39.6 | — |
| 500–999 mAU/mL | 502 (5.8) | 53.1 | 36.5 | — |
| > 1000 mAU/mL | 1525 (17.7) | 48.6 | 33.5 | — |
| Maximal tumor dimension | | | | |
| ≤ 2.0 cm | 2320 (21.0) | 83.7 | 66.3 | 0.0001 |
| 2.1–5.0 cm | 5956 (53.9) | 70.4 | 52.9 | — |
| 5.1–10.0 cm | 1946 (17.6) | 53.0 | 37.5 | — |
| > 10.0 cm | 819 (7.4) | 44.5 | 31.9 | — |
| No. of tumors | | | | |
| 1 | 8412 (75.3) | 73.0 | 56.5 | 0.0001 |
| 2 | 1655 (14.8) | 68.2 | 45.0 | — |
| ≥ 3 | 1108 (9.9) | 44.4 | 26.5 | — |
| Intrahepatic extent of tumor[a] | | | | |
| H0 | 1866 (17.1) | 80.7 | 65.1 | 0.0001 |
| Hs | 3597 (33.0) | 73.6 | 54.9 | — |
| H1 | 2613 (24.0) | 68.3 | 50.3 | — |
| H2 | 2228 (20.4) | 58.3 | 43.0 | — |
| H3–H4 | 604 (5.5) | 36.5 | 26.3 | — |
| Extrahepatic metastasis | | | | |
| Absent | 11,644 (98.7) | 67.7 | 50.9 | 0.0001 |
| Present | 150 (1.3) | 20.5 | 14.9 | — |
| Growth type | | | | |
| Eg | 9936 (92.6) | 69.9 | 52.6 | 0.0001 |
| Ig | 798 (7.4) | 54.8 | 41.8 | — |
| Capsular formation | | | | |
| Absence | 2,670 (24.0) | 70.8 | 55.5 | 0.037 |
| Presence | 8,433 (76.0) | 68.1 | 50.7 | — |
| Septum formation | | | | |
| Absence | 5249 (50.2) | 71.3 | 54.7 | 0.0001 |
| Presence | 5202 (49.8) | 66.7 | 49.7 | — |
| Portal vein invasion | | | | |
| Absence | 8509 (76.5) | 74.9 | 57.2 | 0.0001 |
| Presence | 2609 (23.5) | 48.0 | 34.5 | — |
| Hepatic vein invasion | | | | |
| Absence | 9951 (91.0) | 71.3 | 54.1 | 0.0001 |
| Presence | 983 (9.0) | 44.3 | 33.7 | — |
| Bile duct invasion | | | | |
| Absence | 10,637 (96.6) | 69.8 | 52.9 | 0.0001 |
| Presence | 370 (3.4) | 41.8 | 31.6 | — |
| Surgical curability | | | | |
| Absolute curative | 2239 (20.5) | 81.2 | 65.7 | 0.0001 |
| Relative curative | 5434 (49.7) | 72.5 | 55.6 | — |
| Relative noncurative | 2622 (24.0) | 58.6 | 41.9 | — |
| Absolute noncurative | 649 (5.9) | 35.8 | 19.7 | — |
| Surgical free margin | | | | |
| Presence | 6349 (57.7) | 72.0 | 56.0 | 0.0001 |
| Absence | 4652 (42.3) | 64.3 | 46.7 | — |
| Associated liver disease | | | | |
| Normal | 1064 (11.1) | 69.5 | 58.2 | 0.0001 |
| Chronic hepatitis | 3359 (35.1) | 74.9 | 61.0 | — |
| Cirrhosis | 5142 (53.8) | 65.3 | 46.9 | — |

PIVKA-II: protein induced by vitamin K absence or antagonist-II; Eg: expansive growth (well demarcated border); Ig: infiltrative growth (poorly demarcated border).
[a] H0: a solitary tumor measuring ≤ 2.0 cm in greatest dimension with no vascular invasion; Hs: tumor(s) limited to 1 subsegment (Couinaud segment); H1: tumor(s) limited to 1 segment; H2: tumor(s) limited to 2 segments; H3: tumor(s) limited to 3 segments; H4: tumor(s) involving > 3 segments.

TABLE 3
Multivariate Analysis Using the Stratified Cox Proportional Hazard Model by Associated Liver Disease

| Variable | HR | 95% CI | P value |
|---|---|---|---|
| Age (≥ 60 yrs vs. < 60 yrs) | 1.22 | 1.11–1.33 | 0.0001 |
| Degree of liver damage (C, B, A) | 1.26 | 1.17–1.35 | 0.0001 |
| α-fetoprotein (ng/mL) | | | |
| 21–200 vs. ≤ 20 | 1.35 | 122–1.50 | 0.0001 |
| 201–1000 vs. ≤ 20 | 1.53 | 1.34–1.74 | 0.0001 |
| 1001–10,000 vs. ≤ 20 | 1.57 | 1.36–1.80 | 0.0001 |
| > 10,000 vs. ≤ 20 | 1.64 | 1.38–1.96 | 0.0001 |
| Maximal tumor dimension (cm) | | | |
| 2.1–5.0 vs. ≤ 2.0 | 1.38 | 1.22–1.56 | 0.0001 |
| 5.1–10.0 vs. ≤ 2.0 | 2.04 | 1.75–2.38 | 0.0001 |
| > 10.0 vs. ≤ 2.0 | 2.53 | 2.07–3.09 | 0.0001 |
| No. of tumors (multiple vs. solitary) | 1.19 | 1.05–1.35 | 0.008 |
| Intrahepatic extent of tumor[a] | | | |
| H2 vs. H1 or less | 1.08 | 0.96–1.21 | 0.2 |
| H3/H4 vs. H1 or less | 1.03 | 1.07–1.57 | 0.007 |
| Extrahepatic metastasis (present vs. absent) | 2.19 | 1.55–3.09 | 0.0001 |
| Growth type (Ig vs. Eg) | 1.17 | 0.99–1.38 | 0.06 |
| Capsular formation (present vs. absent) | 1.08 | 0.97–1.38 | 0.17 |
| Septum formation (present vs. absent) | 0.97 | 0.89–1.06 | 0.53 |
| Portal vein invasion (present vs. absent) | 1.46 | 1.31–1.62 | 0.0001 |
| Hepatic vein invasion (present vs. absent) | 1.17 | 1.01–1.36 | 0.03 |
| Bile duct invasion (present vs. absent) | 1.0 | 0.79–1.27 | 0.98 |
| Surgical curability (absolute noncurative vs. others) | 1.4 | 1.18–1.65 | 0.0001 |
| Surgical free margin (positive vs. negative) | 1.1 | 1.01–1.20 | 0.03 |

HR: hazard ratio; 95% CI: 95% confidence interval; Eg: expansive growth (well demarcated border); Ig: infiltrative growth (poorly demarcated border).
[a] H0: a solitary tumor measuring ≤ 2.0 cm in greatest dimension with no vascular invasion; Hs tumor(s) limited to 1 subsegment (Couinaud segment); H1: tumor(s) limited to 1 segment; H2 tumor(s) limited to 2 segments; H3: tumor(s) limited to 3 segments; H4: tumor(s) involving > 3 segments.

prognostic predictors for patients with HCC (Table 3). During the last decade, operative mortality was 2.3% in 1990–1991, 2.0% in 1992–1993, 1.4% in 1994–1995, 1.5% in 1996–1997, and 0.6% in 1998–1999.

## DISCUSSION

The previous LCSGJ report analyzed predictive factors for prognosis of approximately 5800 patients who underwent liver resection for HCC between 1982 and 1989.[2] In the current study, we analyzed a large cohort of > 12,000 patients who underwent liver resection for HCC between 1990 and 1999 in a nationwide survey of primary hepatic cancer in Japan. The development of new diagnostic techniques, such as dynamic computed tomography and magnetic resonance imaging, has lead to an increase in patients who had negative HBs-Ag status with low AFP levels, small tumors, and portal vein invasion compared with our previous report.[2] The number of patients doubled during the last decade, and the patients' profiles have changed. Advances in therapeutic techniques and perioperative patient care have improved long-term outcomes after radical resection for HCC. In the current study, we analyzed patients with HCC in Japan in the 1990s,

during which the 5-year survival rate improved to 50.5% compared with < 40% in the 1980s.[2] The overall cumulative survival rate was better than the rates reported in series from Asian and Western countries.[9,10] Operative mortality also improved to < 1.0% in 1998–1999, compared with > 3.0% in 1982–1987.[2]Therefore, prognostic factors for patients with HCC should be reevaluated.

In our previous study, the univariate analysis showed significant differences for 13 of 14 factors; and multivariate analysis using the Cox proportional hazards model showed that 3 tumor factors (tumor size, number of tumors, portal vein invasion), 3 clinical factors (age, AFP level, and associated liver disease), and 1 operative factor (surgical curability) were independent predictors of long-term prognosis for patients with HCC. In the univariate analysis for the current study, 18 of 21 factors showed significant differences; and the multivariate analysis, which was stratified by associated liver disease, found that 6 tumor factors (tumor size, number of tumors, intrahepatic extent of tumor, extrahepatic metastasis, portal vein invasion, and hepatic vein invasion), 3 clinical factors (age, degree of liver damage, and AFP level), and 2 operative factors (surgical curability and free surgical margin) were independent prognostic factors for overall survival. Thus, tumor size, number of tumors, and portal vein invasion are well known prognostic factors after resection in patients with HCC.

With regard to tumor size, some reports have shown that patients with tumors that measured ≤ 5 cm in greatest dimension had a better prognosis compared with patients who had tumors > 5 cm. A new International Union Against Cancer (UICC) TNM classification system makes use of 5 cm as a tumor cut-off size.[11] However, establishing a screening program for HCC in patients who are at high risk for hepatitis virus infection will increase the diagnoses of small sized HCC tumors. In the current study, the percentage of patients with tumors that measured ≤ 2 cm increased to 21% of all patients, compared with 16% of all patients in our previous report. Three-fourths of patients had tumors that measured ≤ 5 cm. Patients who had tumors that measured ≤ 2 cm had a significantly better prognosis compared with patients who had tumors that measured 2–5 cm. Therefore, in the TNM classification system proposed by the LCSGJ, ≤ 2 cm is the tumor cut-off size.[12]

Hepatic vein invasion was not evaluated as a prognostic factor in the previous study,[2] because only a few patients had hepatic vein invasion. In this large-scale study, however, hepatic vein invasion was an independent prognostic factor, along with portal vein invasion, although the rate of hepatic vein invasion was only 9.0%. Conversely, bile duct invasion was not a predictor in the multivariate analysis, although there were significant differences in survival in the univariate analysis between patients with and without bile duct invasion. Bile duct invasion occurred along with vascular invasion in most patients, which may explain these findings.[13]

Most patients with HCC have HBV or HCV infection. The long-term survival of patients with HCC who have different hepatitis viral infections has been controversial.[14,15] In the current study, there was no significant difference in survival stratified by either hepatitis B or hepatitis C serology; although, in the previous report, patients who had negative HBsAg status had a better prognosis compared with patients who had positive HBsAg status. Conversely, associated liver disease was an important prognostic factor. Patients who had normal livers and chronic hepatitis had a better prognosis compared with patients who had cirrhosis, although there were no significant differences between patients with normal livers and patients with chronic hepatitis. To select adequate therapeutic options for patients with HCC, prognosis should be assessed at the time of preoperative clinical assessment. The degree of liver damage classification, defined by preoperative clinical data similar to the Child–Pugh classification system, was a significant predictor. The degree of liver damage classified by preoperative clinical data was more useful than histologic evaluation of associated liver disease, not only to estimate hepatic functional impairment for determining the appropriate surgical procedure but also to predict patient prognosis.

Serum levels of the tumor markers AFP and PIVKA-II were associated with significant differences in survival. Furthermore, AFP was an independent prognostic factor. We reported previously that AFP and PIVKA II were indicators of a poor prognosis in patients with HCC.[2,3] The Cancer of the Liver Italian Program investigators also reported that AFP was an independent prognostic factor.[16] Koike et al. reported that the PIVKA-II (des-γ-carboxy prothrombin) level was the most useful predisposing clinical parameter for the development of portal vein invasion.[17] However, PIVKA-II was recognized as a useful tumor marker for HCC only in the late 1990s, and it was not examined in 30% of patients in the current study. Therefore, PIVKA-II was not included in our multivariate analysis. Recently, some reports have shown that Lens culinaris agglutinin-reactive AFP is another useful predictor for HCC.[18] These three markers should be evaluated further to determine which tumor markers will be useful in the near future for clinical screening and for determining prognosis in patients with HCC.

Surgical curability was an important prognostic factor. Patients who underwent absolute noncurative resection in which residual tumor remained had a significantly worse survival compared with patients who underwent other types of surgical resection. Llovet et al. reported that the median survival of patients with unresectable HCC who were managed with systematic treatment was 17 months; and their 1-year, 2-year, and 3-year survival rates were 54%, 40%, and 28%, respectively.[19] Those results were similar to the survival results for patients in the current study who underwent absolute noncurative resection. Cytoreduction surgery for HCC does not contribute to improved outcome. Free surgical margin was another important prognostic factor. To avoid tumor recurrence, it is important to perform liver resection with adequate free margins, because microsatellite nodules and histologic venous permeation have been found in adjacent, apparently noncancerous liver.[20]

Overall, within 5 years after they underwent liver resection, 80% of patients developed recurrent disease, and the most common cause of postoperative death was HCC, either due to tumor recurrence or due to multicentric carcinogenesis in the remnant liver. Local ablation therapy and transcatheter arterial chemoembolization in the treatment of recurrent tumors have contributed to improvements in the prognosis for patients with HCC. Liver transplantation is another surgical modality for patients with HCC who have severely impaired liver function, although few Japanese patients underwent liver transplantation in the 1990s. New postoperative adjuvant therapies with interferon may be crucial in reducing the rate of recurrence, especially recurrent multicentric carcinogenesis.[21] However, it remains unknown whether this treatment improves survival.

There are some differences between the recent UICC[11] and LCSGJ TNM staging systems.[12] The major differences between these two TNM staging systems are the tumor cut-off size discussed above and the extent of vascular invasion. Some studies reported that microscopic vascular invasion reflected on prognosis after resection.[22,23] For patients with HCC, several effective, nonsurgical modalities were applied, such as transcatheter arterial chemoembolization, local ablation therapy, etc. Therefore, it is important that patients with HCC select adequate therapeutic options based on a reliable prognostic preoperative assessment using imaging studies and clinical data, and not based on histopathologic reviews of resected specimens. The results of the current study, in which we reevaluated prognostic factors for patients who underwent liver resection using a recent, large-scale data base, will provide useful information with which to evaluate these TNM staging systems. Recently, new staging systems for HCC reflecting tumor status and liver functional status also have been proposed by several groups, such as the Cancer of the Liver Italian Program score,[16] the Barcelona Clinic Liver Cancer stage,[24] and the Japan Integrating Staging score.[25] It will be necessary in the future to establish a common international staging system to guide discussions of treatment for patients with HCC.

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# A subpopulation of bone marrow cells depleted by a novel antibody, anti-Liv8, is useful for cell therapy to repair damaged liver☆,☆☆

Naoki Yamamoto,[a] Shuji Terai,[a,*,1] Shinya Ohata,[b] Tomomi Watanabe,[b] Kaoru Omori,[a] Koh Shinoda,[c] Koji Miyamoto,[d] Toshiaki Katada,[b] Isao Sakaida,[a] Hiroshi Nishina,[b] and Kiwamu Okita[a]

[a] *Department of Molecular Science and Applied Medicine (Gastroenterology and Hepatology), Yamaguchi University School of Medicine, Minami Kogushi 1-1-1, Ube, Yamaguchi 755-8505, Japan*
[b] *Department of Physiological Chemistry, Graduate School of Pharmaceutical Science, University of Tokyo, Hongo 7-3-1, Bunkyoku, Tokyo 113 0033, Japan*
[c] *Department of Neuro-anatomy and Neuroscience, Yamaguchi University School of Medicine, Minami Kogushi 1-1-1, Ube, Yamaguchi 755-8505, Japan*
[d] *Department of Molecular Science and Applied Medicine (Kampo Medicine), Yamaguchi University School of Medicine, Minami Kogushi 1-1-1, Ube, Yamaguchi 755-8505, Japan*

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**Abstract**

We previously reported a new in vivo model named as "GFP/$CCl_4$ model" for monitoring the transdifferentiation of green fluorescent protein (GFP) positive bone marrow cell (BMC) into albumin-positive hepatocyte under the specific "niche" made by $CCl_4$ induced persistent liver damage, but the subpopulation which BMCs transdifferentiate into hepatocytes remains unknown. Here we developed a new monoclonal antibody, anti-Liv8, using mouse E 11.5 fetal liver as an antigen. Anti-Liv8 recognized both hematopoietic progenitor cells in fetal liver at E 11.5 and CD45-positive hematopoietic cells in adult bone marrow. We separated Liv8-positive and Liv8-negative cells and then transplanted these cells into a continuous liver damaged model. At 4 weeks after BMC transplantation, more efficient repopulation and transdifferentiation of BMC into hepatocytes were seen with Liv8-negative cells. These findings suggest that the subpopulation of Liv8-negative cells includes useful cells to perform cell therapy on repair damaged liver.


*Keywords:* Bone marrow cell; Cell therapy; Regenerative medicine; Hepatic stem cell; Migration; Transdifferentiation; Mesenchymal stem cell; Hematopoietic stem cell; Liver regeneration; Niche

Recently, several groups have reported the possible plasticity of bone marrow cells (BMCs) to transdifferentiate into a variety of non-hematopoietic cell lineages [1–4]. Ever since the transdifferentiation of BMC into hepatocytes was documented following a bone marrow transplant from a man donor to a woman recipient [5,6],

☆ *Abbreviations:* BMC, bone marrow cell; $CCl_4$, carbon tetrachloride; FAH, fumarylacetoacetate hydrolase; GFP, green fluorescent protein; EGFP, enhanced GFP; GFP-Tg mice, C57BL6/Tg14 (act-EGFP) OsbY01 mice; HSC, hematopoietic stem cell; E, embryonic day; MSC, mesenchymal stem cells; MAPC, multipotent adult progenitor cell.

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* Corresponding author. Fax: +81-836-22-2240.
*E-mail addresses:* terais@yamaguchi-u.ac.jp (S. Terai), nishina@mol.f.u-tokyo.ac.jp (H. Nishina).
[1] Request for Anti-Liv8 contact to Dr. Hiroshi Nishina, Department of Physiological Chemistry, Graduate School of Pharmaceutical Science, University of Tokyo, Hongo 7-3-1, Bunkyoku, Tokyo 113 0033, Japan.

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BMC has been an attractive cell source in regenerative medicine because getting BMC is easier than obtaining other tissue-specific stem cells [7].

However, the results of recent studies have been mixed in that some studies found that BMC was hardly transdifferentiated while others documented high levels of transdifferentiation [8,9]. Successful transdifferentiation in cell therapy involves various cell and recipient factors, and these factors interact in a complex manner. Therefore, it is difficult to identify the conditions necessary for transdifferentiation, contributing to the varied results among past studies. A past study using a fumarylacetoacetate hydrolase (FAH) knockout mice (metabolic tyrosinemia model) showed that hepatic functions could be compensated by transplanting Lin-Kit+Sca+Thy1low (KTLS) marrow cells [10]. In the FAH model, KTLS cells form foci and transdifferentiate into hepatocytes. Results of recent studies suggest that KTLS cells transdifferentiate into hepatocytes due to fusion with hepatocytes [11,12]. The FAH model is a specialized model of metabolic liver damage, making it possible to analyze the transdifferentiation of BMC into hepatocytes and functional compensation. However, a model with which the transdifferentiation of BMC can be analyzed under conditions of more general liver damage is needed. Using autologous transplantation in GFP transgenic mice [13], we established an isogenic transplantation model to assess the transdifferentiation of BMC into hepatocytes. This model is unique in that uncultured BMCs efficiently migrate into the peri-portal area of the liver and transdifferentiate into immature hepatoblasts and differentiate into mature hepatocytes under the specific "niche" of persistent liver damage induced by persistent intraperitoneal administration of carbon tetrachloride ($CCl_4$) [14]. In this model, liver cirrhosis was induced by 4 weeks $CCl_4$ injection, and BMCs isolated from GFP transgenic mice were transplanted through the caudal vein. It is possible to chronologically observe colonization and transdifferentiation of BMC in the liver by continuous administration of $CCl_4$, and we have named this model as the "GFP/$CCl_4$ model." Furthermore, in this model, as in the natural development of the liver, BMCs appear to be transdifferentiated into hepatoblasts and then into hepatocytes. In our GFP/$CCl_4$ model, the timing of cell transplantation and the state of recipients appear to be suitable for the transdifferentiation of BMC into hepatocytes. Cell transplantation and continuous liver damage made efficient transdifferentiation of BMC into hepatocytes. In a system similar to ours, human hematopoietic stem cells (HSCs) were transplanted into the bone marrow of immunologically tolerant NOD/SCID mice before administration of $CCl_4$, and these cells differentiated into albumin-positive hepatocyte-like cells after the $CCl_4$ administration [15]. These findings suggest that a special "niche" created by $CCl_4$-induced liver damage is important for the migration of BMC to the liver and transdifferentiation into hepatocytes. Also, it has been reported recently that $CCl_4$ administration is effective for improving the colonization of HSC to liver of NOD/SCID [16].

The liver functions as a metabolic organ, but during the fetal period, from embryonic day (E) 12 to 16 (E12–E16), the liver functions as a hematopoietic organ [17]. Several studies have reported that mesenchymal cells affect hepatic hematopoiesis during the fetal period [18,19]. After this hematopoietic period, hepatoblasts are involved in a complex manner to develop the liver as a metabolic organ. However, documentation of the existence of HSC in the adult liver suggests that, even in the adult liver, blood cells and hepatocytes still play some role in the maintenance of hepatic function [20]. To further analyze this aspect, we prepared new rat monoclonal antibodies using the fetal liver on E 11.5 as an antigen. One of these antibodies, anti-Liv2, specifically recognizes hepatoblasts in the fetal liver from E 9.5 to 12.5. The results of past studies using the anti-Liv2 antibody have shown that SEK1, a stress-signaling kinase, plays an important role in the proliferation of hepatoblasts, thus suggesting that inflammatory signals are involved in the proliferation of hepatoblasts [21].

Although various theories explain the existence of pluripotent stem cells in BMC, the exact composition of stem cells in BMC is not clear at this time; the following cell types are known to exist in bone marrow: HSC [4,10], side population cells [22], and mesenchymal stem cells (MSC) [23]. Although past studies used the existing antibodies and techniques, there have not been any studies based on the findings associated with natural liver development. Using fetal liver as an antigen, we prepared a new monoclonal antibody, anti-Liv8 antibody, to analyze which subpopulation of BMC could differentiate into hepatocytes under $CCl_4$-induced continuous liver damage in the GFP/$CCl_4$ model [14]. This anti-Liv8 antibody recognizes hematopoietic cells using a specific cell surface marker and it can be used to separate cells. In the present study, we used this new antibody to separate BMC of adult mice and then transplanted the different types into mice under identical conditions of the GFP/$CCl_4$ model to ascertain which types of BMCs transdifferentiate into hepatocytes.

## Materials and methods

*Mice.* C57BL6/Tg14 (act-EGFP) OsbY01 mice (GFP-Tg mice) showed GFP expression in multiple tissue and cells and were kindly provided by Masaru Okabe (Genome Research Center, Osaka University, Osaka, Japan) [13]. C57BL/6 female mice were purchased from Japan SLC (Shizuoka, Japan). AML1 knockout mice were generated

as described previously [24]. The genetic background of these mice used in this study was C57BL/6 mice. Male and female mice were mated overnight and female mice were scored based on vaginal plaques taken to represent E 0.5. Mice were anesthetized at the completion of experiments. All processes, including surgical steps, were undertaken with the guidance of the committee for animal and recombinant DNA experimentation at Yamaguchi University.

*Production of rat monoclonal antibody, Liv8.* Eight-week-old WKY/NCrj female rats were immunized in the hind footpads with 100 μg E 11.5 murine fetal liver lysate in complete Freund's adjuvant (0.2 ml). Anti-Liv8 antibodies were raised according to a previously described protocol [21].

*Immunohistochemical staining for fetal liver.* Fetal liver at E11.5 was obtained from c57/BL/6 mice and AML1 knockout mice. Tissue preparation and immunohistochemical analysis were performed according to a previously described protocol [21]. We analyzed anti-Liv2- and anti-Liv8-positive cells in fetal liver.

*Preparation of GFP-positive BMC.* For isolation of BMC, GFP-Tg mice were sacrificed by cervical dislocation and the limbs were removed. GFP-positive BMCs were flushed from the medullary cavities of tibias and femurs with PBS culture solution using a 25 G needle. The cell solution was filtered through a cell strainer (16 μm) to remove particular matter and centrifuged at 500*g* for 5 min. After centrifugation, the supernatant was removed and cells were resuspended to prepare $1.0 \times 10^6$ cells/ml GFP-positive BMC solutions. Preparation of BMC takes approximately 1.5 h.

*FACS analysis of BMC using Liv8 antibody.* Prepared GFP-positive BMCs were reacted with rat biotin anti-Liv8 IgG antibody, R-Phycoerythrin (R-PE)-conjugated rat anti-CD45 (leukocyte common antigen) monoclonal antibody (PharMingen, San Diego, USA) at the rate of 1 μg per $10^6$ total cells, mixed well, and incubated in the gobos for 30–40 min at 4 °C. Following the incubation with the first antibody, the cells were washed twice by 0.02 M PBS and centrifuged at 500*g* for 5 min. Labeled cells were then reacted to streptavidin–fluorescein isothiocyanate (FITC) conjugate (PharMingen) at the rate of 1 μg per $10^6$ total cells, mixed well, and incubated in the gobos for 30–40 min at 4 °C. After that, these were washed out once with 0.02 M PBS and centrifuged at 500*g* for 5 min. The labeled cells were analyzed using FACS Calibur (Becton–Dickinson).

*Sort GFP positive BMC by Liv8 antibody.* Prepared BMCs were reacted to rat anti-Liv8 IgG antibody at the rate of 1 μg per $10^6$ total cells, mixed well, and incubated in the gobos for 30–40 min at 4 °C. Then cells were washed two times by 0.02 M PBS and centrifuged at 500*g* for 5 min. Cells were labeled with rat anti-Liv8 IgG antibody by reacting with Goat Anti-Rat IgG MicroBeads (Miltenvi Biotec GmbH, Bergisch Gladbach, Germany) at the rate of 20 μl per $10^7$ total cells, mixed well, and incubated for 20–30 min at 4 °C. Labeled cells were washed once by 0.02 M PBS and centrifuged at 500*g* for 5 min. These cells were separated into Liv8-positive cells or negative cells by the Auto Magnetic Cell Sorting system (Auto MACS) (Miltenvi Biotec GmbH) for 10 min per tube.

*Transplantation of Liv8-positive or negative BMC into persistent liver damaged mice.* We developed a new in vivo model "GFP/$CCl_4$ model" for monitoring differentiation of BMCs into hepatocytes [14]. To generate a liver damage group, 0.5 ml/kg of $CCl_4$ was injected into the peritoneum of 6-week-old C57BL/6 females twice a week for 4 weeks. Liver cirrhosis resulting from the continuous injections of $CCl_4$ was confirmed. A control group of C57BL/6 mice that had not been treated with $CCl_4$ was also used. One day after the eighth injection, sorted Liv8-positive or Liv8-negative BMC ($1 \times 10^5$ cells) was slowly injected into the caudal tail vein of mice using a 31 G needle and a Hamilton syringe. After transplantation, $CCl_4$ injections (0.5 ml/kg) were continued twice a week. Mice were sacrificed weekly up to 4 weeks.

*Tissue preparation.* The livers were thoroughly perfused via the heart with 4% paraformaldehyde (Muto, Tokyo, Japan). This step was crucial for washing out contaminating blood cells. For fixation, the perfused livers were incubated with 4% paraformaldehyde (Muto) overnight and then soaked in 30% sucrose for a few more 3 days. Tissues were frozen in dry ice and then sectioned into 18-μm slices using a cryostat (Moriyasu Kounetsu, Osaka, Japan) in preparation for dyeing.

*Immunohistochemistry and double immunofluorescence for GFP.* To avoid autofluorescence, we used immunostaining to assess the expression of GFP. Cells expressing GFP were analyzed by both fluorescent microscopy and conventional immunohistochemistry with anti-GFP antibody (Santa Cruz Biotechnology, Santa Cruz, California, USA). Immunohistochemical analysis was performed according to a previously described protocol [14,25]. Sectioned tissues were incubated with anti-GFP antibody (1:5000 FL, sc-8334; Santa Cruz Biotechnology), anti-albumin (1:5000, 55462; ICN Pharmaceuticals, Costa Mesa, CA, USA), and anti-Liv2 antibody (1:5000) [21]. For fluorescence immunohistochemistry, tissues were incubated with Alexa Fluor R 488 and 568 donkey anti-goat IgG(H + L) conjugate, Alexa Fluor R 488 goat anti-rabbit IgG(H + L) conjugate, and Alexa Fluor R 568 goat anti-rat IgG(H + L) conjugate (Molecular Probes, Eugene, OR) as secondary antibodies. Positive cells in the liver were quantified using a Provis microscope (Olympus, Tokyo, Japan) equipped with a charge coupled devise (CCD) camera and subjected to computer-assisted image analysis with MetaMorph software (Universal Imaging, Downingtown, PA). A total of 10 different areas per liver section were analyzed independently and the areas of positive cells were calculated using the MetaMorph software.

*Serum albumin level analysis.* Serum albumin levels during the 4 weeks after Liv8-positive or Liv8-negative BMC transplantation were analyzed using the SPOTCHEM EZ SP-4430 dry chemical system (Arkray, Kyoto, Japan).

*Statistical analysis.* Values are shown as means ± SE. Data were analyzed by analysis of variance with Fisher's projected least significant difference test.

## Results

### *Anti-Liv8 antibody detected hematopoietic progenitor cell in fetal liver at E 11.5*

Previously we had raised a rat monoclonal antibody, anti-Liv2, which recognized hepatoblasts at E 9.5 [21]. As shown in Fig. 1A, Liv2-positive cells were also detected in fetal liver at E 11.5. Using the antibody developed in this study, Liv8-positive cells were seen in the fetal liver on E 11.5 (Fig. 1B). Fetal liver at E 11.5 functions as a secondary hematopoietic organ [17]. We analyzed whether anti-Liv8 positive cell is associated with hepatoblast or hematopoietic cell. We found Liv2-positive cells (Fig. 1C), but no Liv8-positive cells (Fig. 1D), in the fetal liver of $AML1^{-/-}$ embryos which do not undergo definitive hematopoiesis [24]. These results suggested that anti-Liv-8 recognizes hematopoietic progenitor cell in fetal liver.

### *Liv8-positive cells exist in adult bone marrow and express CD45*

Next, we investigated Liv8-positive cells in the BMC of adult GFP Tg mice. Liv8-positive cells were found to be present among adult BMCs in adult bone marrow when analyzed in GFP-Tg mice. We found around 32%

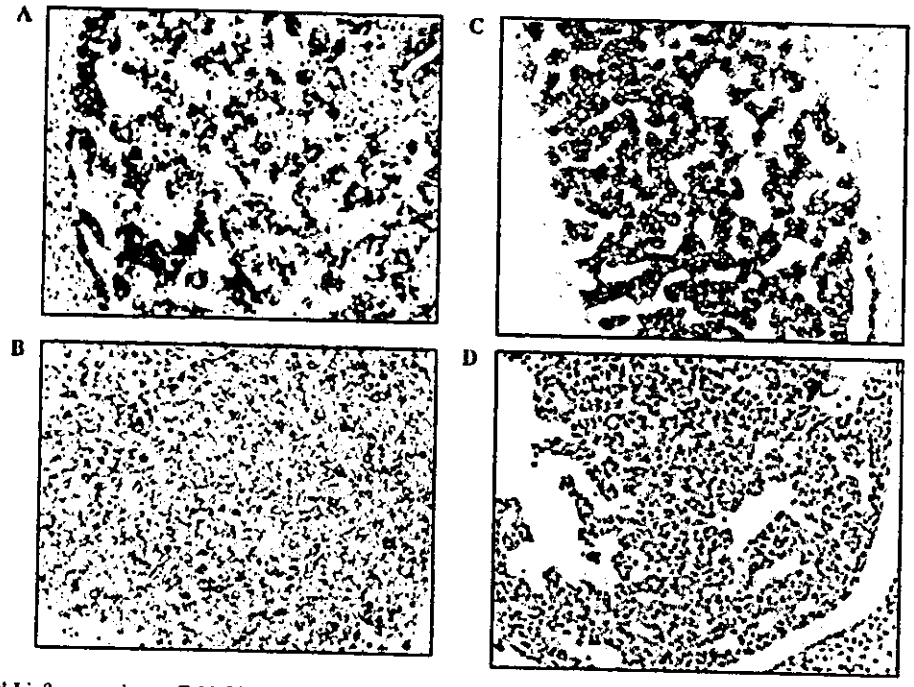


Fig. 1. (A–D) Liv2 and Liv8 expression at E 11.5 in normal and AML1$^{-/-}$ mice. Liv2 (A,C) and Liv8 (B,D) expression at E 11.5 in normal fetal liver (A,B) and AML1$^{-/-}$ mice (C,D). Magnification: (A–D) at 200×.

of Liv8-positive cells in adult GFP-Tg mice (Fig. 2A). We also analyzed the relationship between Liv8 and CD45, and found that 54% of Liv8-positive cells also expressed CD45 (Fig. 2B). These results showed that anti-Liv8 is useful to separate hematopoietic cell and non-hematopoietic cell.

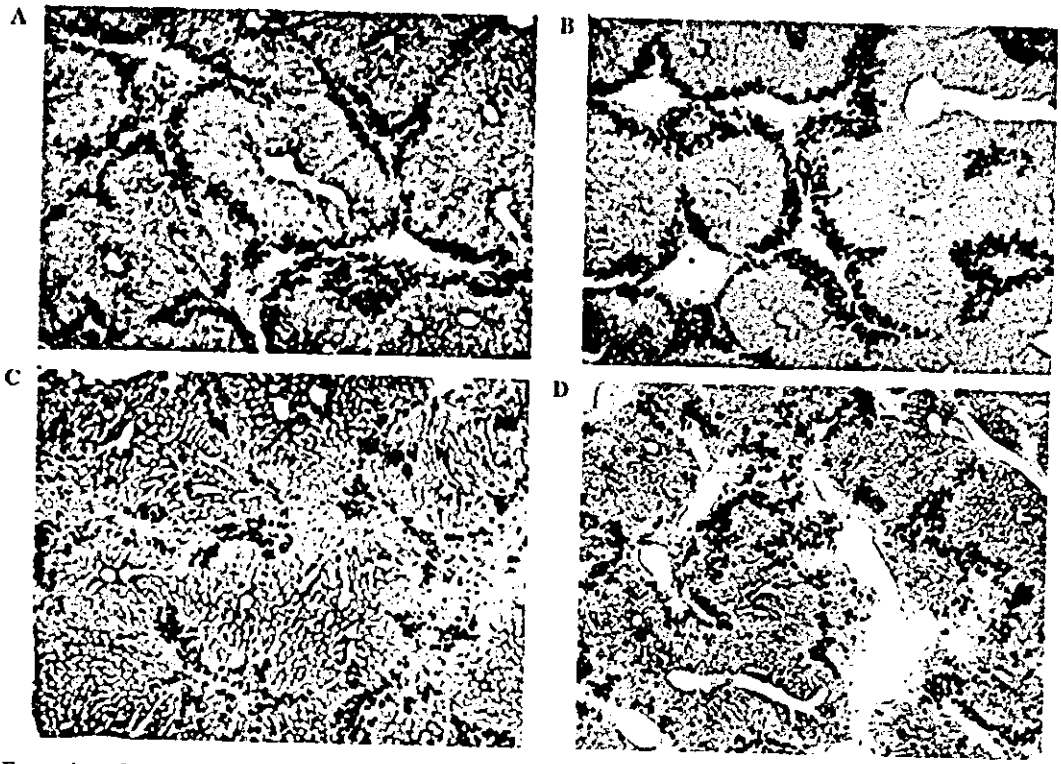


Fig. 3. (A–D) Expression of GFP in liver after transplantation of Liv8-positive and Liv8-negative cells. GFP expression in the liver after transplantation of Liv8-positive BMCs at 1 week (A) and 4 weeks (C), GFP expression at the liver after Liv8-negative BMC transplantation at 1 week (B) and 4 weeks (D) after cell injection. Magnification 200×.

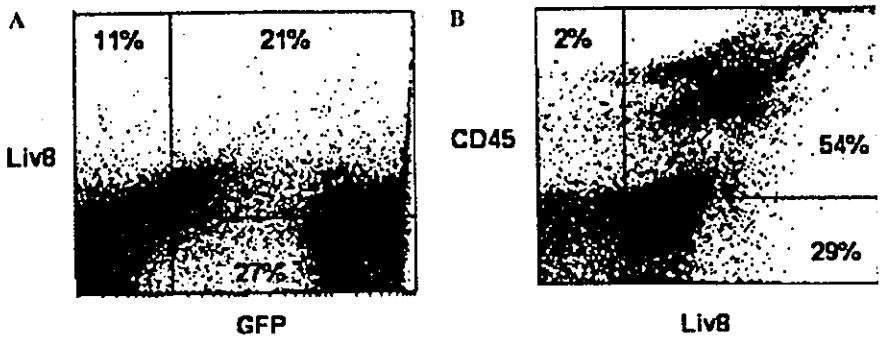


Fig. 2. Expression of CD45, Liv8 in bone marrow cell. FACS analysis of all BMCs of GFP-Tg mice. (A) Staining with Liv8 and GFP. (B) Staining with CD45 and Liv8.

*Liv8-negative cells repopulated at the liver more than Liv8-positive cells*

After separating Liv8-positive cells from Liv8-negative cells using AutoMACS, these cells were transplanted to recipient mice with $CCl_4$-induced liver cirrhosis. At one week after transplantation, both Liv8-positive (Fig. 3A) and Liv8-negative cells (Fig. 3B) colonized around the portal vein, with no marked differences in the rate of colonization (Table 1). In the Liv8-positive cell transplanted group, the number of GFP-positive cells in the liver increased transiently, but at four weeks after transplantation, the number of GFP-positive cells was significantly lower in the Liv8-positive cell group (Fig. 3C) than in the Liv8-negative cell group (Fig. 3D). Furthermore, GFP-positive cells were colonized inside the hepatic lobes in the Liv8-negative cell group at four weeks after transplantation. These results showed that Liv8-negative cell repopulated more than Liv8-positive cell.

*The Liv8-negative cells transdifferentiate into hepatoblast phenotype*

We showed in previous studies that transplanted BMCs transdifferentiate into Liv2-positive hepatoblasts and then further differentiate into hepatocytes [14,21]. In the present study, we also investigated the presence of cells expressing Liv2. Liv2-positive cells were identified by immunostaining, and the results showed that Liv2-positive cells were seen around the portal region one week after transplantation, but that there was no significant difference in the number of Liv2-positive cells between Liv8-positive and Liv8-negative cell groups (Figs. 4A and B, and Table 1). With time, the number of Liv2-positive cells in the liver decreased significantly for the Liv8-positive cell group (Figs. 4C and D and Table 1). The transdifferentiation of myelogenic GFP cells into Liv2 cells was investigated. Cells that expressed both Liv2 and GFP were detected at four weeks after transplantation, and fluorescent staining showed that the expression of Liv2 by myelogenic cells was higher for the Liv8-negative cell group (Figs. 4E and F). These results indicated that Liv8-negative cell could be transdifferentiated into hepatoblast phenotype.

*Albumin expression in the liver and serum albumin level following transplantation of Liv8-positive and Liv8-negative BMCs*

At one week after cell transplantation, there was no marked change in the expression of albumin for both Liv8-positive and Liv8-negative cell groups (Figs. 5A and B). However, at four weeks after transplantation, the expression of albumin decreased with time for the Liv8-positive cell group (Fig. 5C), but remained the same for the Liv8-negative cell group (Fig. 5D). Furthermore, at four weeks after cell transplantation, the number of yellow cells expressing both albumin and GFP was higher for the Liv8-negative cell group

Table 1
Percent of area for each differentiation marker after Liv8(+) and Liv8(−) cell transplantation under the persistent liver damage

| | | 1 week ($n = 5$) | 2 weeks ($n = 5$) | 3 weeks ($n = 5$) | 4 weeks ($n = 5$) |
|---|---|---|---|---|---|
| GFP | Liv8(+) | 11.1 ± 1.7 | 15.1 ± 2. 1 | 9.4 ± 0.8 | 5.1 ± 0.6* |
| | Liv8(−) | 11.7 ± 1.0 | 13.2 ± 0.8 | 12.4 ± 2.6 | 9.5 ± 3.6* |
| Liv2 | Liv8(+) | 6.0 ± 1.1 | 7.3 ± 3.5 | 8.2 ± 1.8 | 3.3 ± 0.9 |
| | Liv8(−) | 5.5 ± 1.3 | 5.8 ± 0.8 | 9.2 ± 0.6 | 7.7 ± 0.9 |
| Albumin | Liv8(+) | 15.0 ± 1.9 | 14.9 ± 2.5 | 6.8 ± 2.6* | 3.7 ± 1.4* |
| | Liv8(−) | 12.7 ± 3.2 | 12.5 ± 3.2 | 14.8 ± 1.3* | 10.6 ± 2.1* |

Values shown are percent of the area occupied.
*showed significant differences at each sampling point ($n = 5$) at $p < 0.05$ between Liv8(+) and Liv8(−) cell transplantation groups.

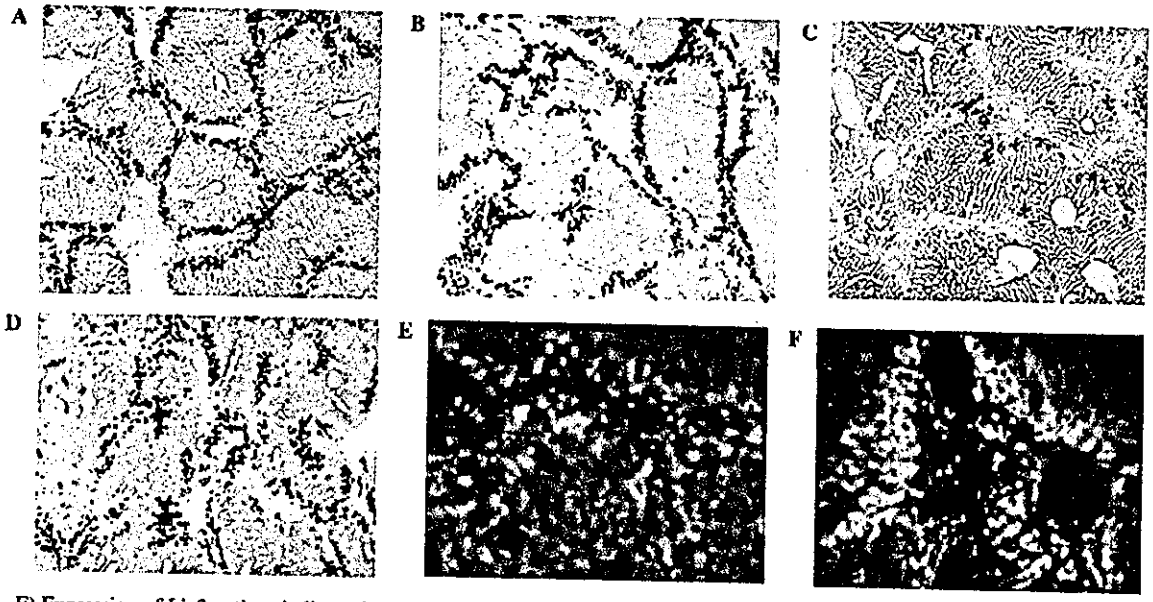


Fig. 4. (A–F) Expression of Liv2 antigen in liver after transplantation of Liv8-positive and Liv8-negative cells. Liv2 antigen expression at 1 week (A) and 4 weeks (C) after Liv8-positive BMC transplantation. Magnification at 200×. Liv2 antigen expression at liver at 1 week (B) and 4 weeks (D) after Liv8-negative BMC transplantation. Double fluorescent staining (red, Liv2; green, GFP; and yellow, Liv2 & GFP) of the liver at 4 weeks after Liv8-positive cell transplantation (E) and Liv8 negative cell transplantation (F) Magnification: (A–D) 200×, (E,F) 400×.

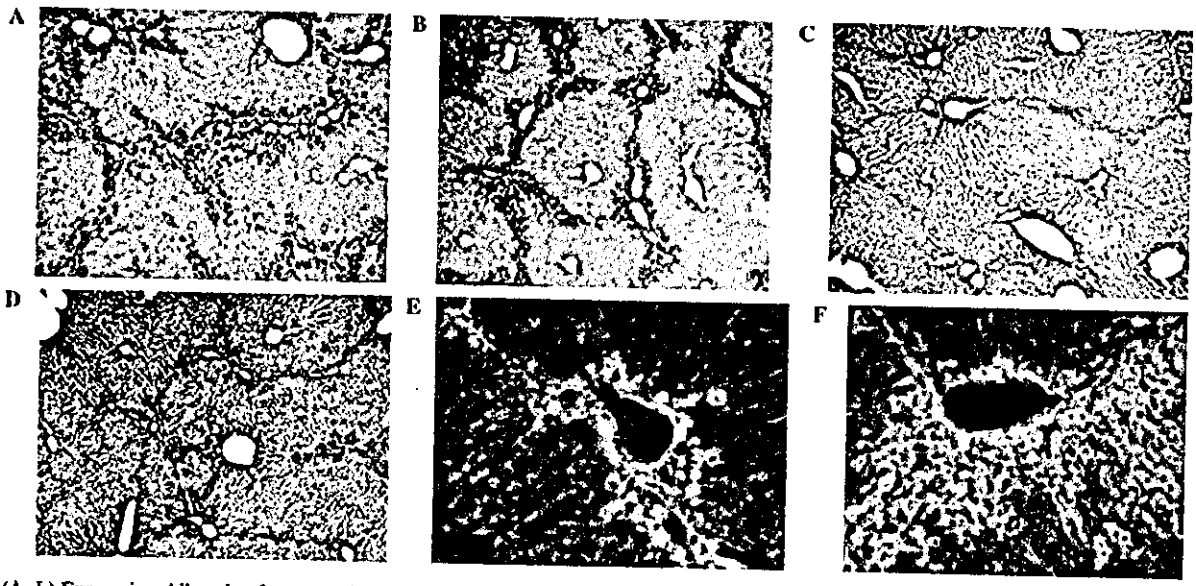


Fig. 5. (A–L) Expression Albumin after transplantation of Liv8-positive and Liv8-negative cells. Albumin expression at 1 week after transplantation of Liv8-positive cells (A) and Liv8-negative cells (B). Albumin expression at 4 weeks after transplantation of Liv8-positive cells (C) and Liv8-negative cells (D). Double fluorescent staining (red, albumin; green, GFP; and yellow, albumin & GFP) of liver at 4 weeks after transplantation of Liv8-positive cells (E) and Liv8-negative cells (F). Magnification: (A–D) 200×, (E,F) 400×.

(Figs. 5E and F). To ascertain whether transplanted cells were functioning as hepatocytes, serum albumin levels were measured. Serum albumin levels increased for both groups and were higher for the Liv8-negative cell group than the Liv8-positive cell group. The serum albumin levels at 4 weeks after Liv8-negative BMC transplantation showed the significantly higher levels for Liv8-negative cell group compared to the Liv8-positive BMC group ($n = 5$, $p < 0.05$) (Fig. 6). These results also showed that Liv8-negative cell could transdifferentiate into albumin-positive hepatocyte.

## Discussion

The anti-Liv8 antibody is a useful antibody to separate hematopoietic cells and non-hematopoietic cells in adult bone marrow. We found Liv8-positive cells in fetal

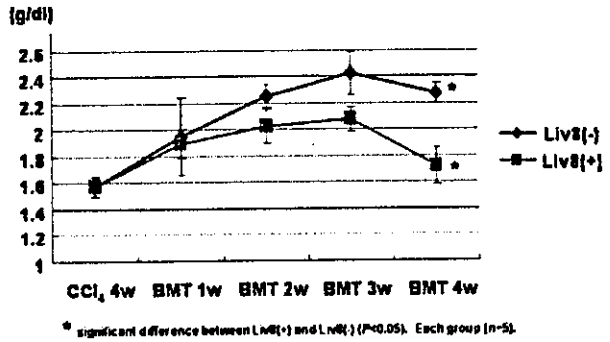


Fig. 6. The level of serum albumin. Serum albumin levels after Liv8-positive or Liv8-negative cell transplantation. $CCl_4$ 4w, 4 weeks $CCl_4$ injection group. BMT 1w, 1 week after BMC transplantation. BMT 2w, 2 weeks after BMC transplantation. BMT 3w, 3 weeks after BMC transplantation. BMT 4w, 4 weeks after BMC transplantation. * showed significant differences at each sampling point ($n = 5$) at $p < 0.05$.

liver at E11.5, but could not detect no-positive cells in fetal liver of AML1 knockout mice (Fig. 1C) at E 11.5. This result suggested that anti-Liv8-positive cell might be associated with the generation of HSC. We used FACS analysis to understand more about the characterization of Liv8-positive cells in the bone marrow. Around 32% of all BMCs, which were positive for Liv8, also expressed CD45 (Figs. 2A and B). CD45 is the pantrophic marker for hematopoietic cell marker [26,27]. These results suggest that anti-Liv8 recognizes most hematopoietic cells. We separated BMCs into Liv8-positive cells and Liv8-negative cells using Auto-MACS, and the repopulation and transdifferentiation of these cells into liver was analyzed in the GFP/$CCl_4$ model [14].

First we analyzed the colonization of transplanted Liv8-positive or negative cell. There was no change in the ratio of GFP-positive cells one week after transplantation between the Liv8-positive and Liv8-negative cell groups (Figs. 3A and B). In both groups, GFP-positive cells were found around the portal vein. The expression of GFP decreased with time for the Liv8-positive cell group (Fig. 3C), but in the Liv8-negative cell group, GFP-positive cells entered the hepatic lobes (Fig. 3D). At four weeks after transplantation, the rate of colonization for the Liv8-positive cell group was significantly lower than that for the Liv8-negative cell group (Table 1). Previously we found that colonization was not observed when BMCs were transplanted to normal recipients, but colonization was observed when BMCs were transplanted to recipients with liver cirrhosis caused by administration of $CCl_4$ [14]. Some previous studies also have reported that $CCl_4$ injection enhances the repopulation of hepatocytes following hepatocyte transplantation via the spleen [28,29]. It has been documented that elevated levels of SDF1 and matrix metalloprotease 9 (MMP9) might have an important role for the migration of BMCs to the liver at liver damage by $CCl_4$ administration [16,30]. In the GFP/$CCl_4$, the expression of MMP9 was also increased by the transplantation of BMCs (I. Sakaida, unpublished data). At 1 week after transplantation, there was no marked difference in colonization between the Liv8-positive and negative transplantation groups. These results suggest that the early migration of BMC into liver was determined by the recipient condition. Next we analyzed the transdifferentiation of BMC into functional hepatocyte in the "niche" where transdifferentiation of BMC into hepatocyte is favorable [14]. The results of our past analyses have shown that transplanted BMCs transdifferentiate into Liv2-positive hepatoblasts and then differentiate into hepatocytes only under continuous inflammation. The persistent liver damage made by injection of persistent $CCl_4$ injection is important for the transdifferentiation of BMC [14]. When human HSCs were transplanted to immunologically tolerant NOD/SCID mice and followed up with administration of $CCl_4$, it was found that transplanted human HSC was differentiated into albumin express hepatocyte-like cell [15]. Albumin/promoter-Alb-DsRed2 Tg rat was established to monitor the transdifferentiation into albumin positive cell. Albumin-producing DsReds cell was increased by repeated administration of $CCl_4$ [31]. A study reported recently that the transdifferentiation of BMCs was low when inducing liver damage by $CCl_4$ administration before or after transplantation [32]. Different results were obtained with these systems because chronic liver damage before and after transplantation was not evident. The persistent liver damage might be the key factor to induce the transdifferentiation of BMC into hepatocyte. We investigated the transdifferentiation of Liv8 positive and negative BMCs into hepatoblast and hepatocytes by Liv2 and albumin expression. Like GFP, Liv2-positive cells were seen around the portal vein one week after transplantation for both Liv8-positive and Liv8-negative cell groups, and there was no marked difference between the two groups (Figs. 4A and B). On the other hand, at four weeks after transplantation, the expression of Liv2 for the Liv8-positive cell group was significantly lower than that for the Liv8-negative cell group (Figs. 4C and D). The results of double staining at four weeks after transplantation also showed that the number of myelogenic Liv2-positive cells was greater for the Liv8-negative cell group (Figs. 4E and F). Figs. 5C and D show the expression of albumin four weeks after transplantation and the expression of albumin for the Liv8-negative cell group was higher (Fig. 5D). The expression of albumin and GFP in myelogenic cells was significantly higher for the Liv8-negative cell group (Fig. 5F). Furthermore, we investigated functional recovery by comparing improvement in hepatic failure between the Liv8-positive and Liv8-negative cell groups.

As shown in Fig. 6, when $CCl_4$ was administered in the same manner to the Liv8-positive and Liv8-negative cell groups, and the level of serum albumin increased in both groups, but a significant finding in this analysis was significant improvements in the serum albumin levels at four weeks after transplantation in the Liv8-negative cell group compared to the Liv8-positive cell group ($p < 0.05$). These findings support those of immunostaining. These results can be summarized that Liv8-negative cells are more likely to transdifferentiate into hepatocytes with time passed. The subpopulation which was deleted by anti-Liv8 will be useful cells to use cell therapy using BMC to repair damaged liver. The Liv8 negative cell was thought to be non-hematopoietic cells. For example, multi-potent adult progenitor cells (MAPCs) from BMCs differentiate into functional hepatocyte like cells [33,34]. Our results might support that mesenchymal cells may differentiate into pluripotent cells under certain conditions.

Still the precise mechanisms to regulate repopulation and transdifferentiation BMC into hepatocyte are uncertain. To develop a cell therapy using BMC to repair damaged liver, we are planning to further analyze these mechanisms.

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# Herbal medicine Sho-saiko-to (TJ-9) increases expression matrix metalloproteinases (MMPs) with reduced expression of tissue inhibitor of metalloproteinases (TIMPs) in rat stellate cell

Isao Sakaida[a,*], Koji Hironaka[a], Teruaki Kimura[b], Shuji Terai[a], Takahiro Yamasaki[a], Kiwamu Okita[a]

[a] *Department of Gastroenterology and Hepatology, Yamaguchi University, School of Medicine, Minami-Kogushi 1-1-1, Ube, Yamaguchi 755-8505, Japan*
[b] *Department of Bioregulatory Function, Yamaguchi University, School of Medicine, Minami-Kogushi 1-1-1, Ube, Yamaguchi 755-8505, Japan*

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**Abstract**

We have reported that Sho-saiko-to (TJ-9) prevents liver fibrosis in vivo. To gain further insights into the effect of TJ-9, the matrix metalloproteinases (MMPs)/tissue inhibitors of metalloproteinases (TIMPs) balance was examined. Hepatic stellate cells (HSCs) were isolated from male Wistar rats and cultured with TJ-9 (0-1000 μg/ml) on uncoated plastic dishes for 4 days. To elucidate the effects on the MMPs/TIMPs balance by TJ-9, quantitative analysis of type IV collagen-degrading activity, gelatin zymography and reverse zymography were carried out. Northern blot analysis was performed to determine the expression of MMP-2, 13 and TIMP-1 mRNAs. TJ-9 treatment resulted in dose-dependent upregulation of MMP-2, 13 mRNA and downregulation of TIMP-1 mRNA up to 500 μg/ml. Gelatin zymography, reverse zymography and quantitative analysis of type IV collagen-degrading activity confirmed that TJ-9 increased MMP-2 activity and prevented TIMP-1, 2 activities in a dose-dependent manner. SB203580 diminished the reduction of mRNA as well as the activity of TIMP-1 by TJ-9 and induction of mRNA as well as the activity of MMP-2. These results show that TJ-9 increased MMP-2, 13 activity with reduced TIMP-1, 2 activities on HSCs possibly via P38 pathway.


*Keywords:* Herbal medicine; Fibrosis; Signal transduction; Metalloproteinase; Tissue inhibitor of metalloproteinase

* Corresponding author. Tel.: +81-836-22-2241; fax: +81-836-22-2240.
*E-mail address:* sakaida@po.cc.yamaguchi-u.ac.jp (I. Sakaida).

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